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14. ABSTRACT

We proposed to study the mechanisms of androgen resistance by focusing on androgen receptor mutations that may arise due to selective pressures of antiandrogen treatment. We have utilized xenograft models and a humanized mouse model (h/mAR xTRAMP) of prostate cancer. Novel AR mutations were found throughout the entire coding region of AR although they did not segregate into distinct regions based on hormonal treatment as was previously reported. Functional analysis of some of these mutations was carried out. Preliminary analysis suggests some AR variants show differential action on different promoters and in different cell types suggesting promoter and cell specific effects. Interestingly a high number of mutations occurred in treated mice when compared to non treated mice supporting a role for treatment in AR variant generation. Analysis of tumor progression in the h/mAR X TRAMP mice have highlighted differences in disease course between antiandrogen treated and hormone deplete (castrated) mice. Utilization of m/hARxTRAMP mice provided us with a tool to better understand the mechanisms of androgen resistance in prostate cancer and will aid research into more effective treatments.

15. SUBJECT TERMS
Androgen receptor, Androgen independent disease, Flutamide and bicalutamide

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INTRODUCTION

Androgens play a critical role in prostate growth and differentiation and this hormone dependence has been utilized extensively in the treatment of localized and advanced prostate cancer. Prostate Cancer (PCa) therapy involves the withdrawal of androgen and/or the inhibition of androgen receptor function (Brawer, 2006). While tumors regress initially, failure of endocrine therapy occurs and tumor growth is initiated ultimately leading to a fatal outcome.

An important feature, common to all prostate cancers, is the presence of the androgen receptor at all stages of tumor progression (Tilley et al 1996). Significantly, the androgen receptor signalling axis appears active; as AR regulated genes, such as prostate specific antigen (PSA), continue to be expressed in the advanced disease state. Therefore, PCa appears to be androgen independent but androgen receptor dependent.

Suggested mechanisms of androgen resistance include androgen receptor amplification, ligand independent AR activation, androgen receptor mutation, as well as, aberrant activation of growth signaling pathways that are independent of androgen receptor action. This study aims to addresses the role of androgen receptor mutation in the attainment of androgen resistance. We propose that the selection of mutant ARs maybe treatment specific. We suggest that genetic alterations in AR occur as a direct consequence of the specific endocrine selection pressures applied to the prostate tumor during androgen ablation therapy

BODY

The primary goal of this proposal was the investigation of mechanisms of androgen resistance in prostate cancer, with particular emphasis on the actions of the antiandrogens, bicalutamide and flutamide. We hypothesized that tumor progression may be due, in some cases, to variant androgen receptor arising due to selection pressure conferred by antiandrogens. It is also proposed that the antiandrogens, bicalutamide and flutamide differ in their precise mechanisms and therefore, any alterations to androgen receptor structure and/or function may vary with the particular antagonist.

To test our hypothesis that antiandrogen therapy can contribute to tumor prostate progression via genetic or epigenetic alterations in the androgen receptor (AR) we proposed to utilise prostate cell lines and xenograft models. We anticipated analyzing the mechanistic actions of bicalutamide and flutamide in prostate cancer cells *in vitro* using transfection analysis in a variety of prostate cell lines and using different promoter constructs (Task1). To analysis the potential selective pressure of antiandrogen therapy on the production of AR variants we proposed to sequence AR cDNA from xenograft prostate cancer tumors that were removed from SCID mice treated with androgen ablation therapy (Task 2). Mutations identified by sequence analysis will be tested functionally in vitro (Task 3)

Task 1- Mechanistic actions of bicalutamide and flutamide in vitro

Rationale

An initial aim was to examine potential differential actions of biculatamide and flutamide on the transactivation of androgen responsive genes. Advanced prostate cancer patients often respond to a second line of antiandrogen therapy (Miyake et.al. 2005). It has also been illustrated that antiandrogens can have differing effects on the interactions between AR and co-activator molecules (Song et al., 2004). This suggests that different antiandrogens may have alternate downstream signaling mechanisms, which may ultimately result in differential modes of prostate disease progression. Our hypothesis suggests that bicalutamide and flutamide will have subtly different effects on AR function that may be accentuated by different promoter and cell-context effects.

Experimental Design

It was expected that we would focus our initial studies on PC-3 cells with CV-1 cells as a experimental control, and later extend our experiments to include the LNCaP cell line, two PC-3 sublines, PC-3^{AR} (containing the stably transfected human AR) and PC-3^{LNCaP} (containing the stably transfected mutated LNCaP AR [T877A]) and the cell lines, C1, C2, and C3 isolated from the TRAMP mouse model. It was proposed that utilization of this panel of cells will provide information of cell-context specific effects as well as species-specific effects of antiandrogens action. Furthermore comparison of different promoter responses within a particular cell type will indicate if any promoter-specific effects play a role in antiandrogen action.

Results/Discussion

To explore potential cell context effects on antiandrogen action we initially focused on two androgen receptor negative cell lines, CV-1 and PC 3. CV-1, a monkey kidney fibroblast cell line used extensively in transfection studies (Jensen et al 1964) while the PC3 cell line is representative of an advanced prostatic carcinoma isolated from a lumbar vertebral metastasis. The PC3 cell line has been used widely in the field of prostate cancer and exhibits androgen independent tumor growth both *in vitro* and in *vivo* (Kaighan et al 1974).

Both cell lines were transfected with 4 ng pSG5 human androgen receptor expression vector and 20 ng CMV–renilla vector which was used as a transfection control. Two contrasting target reporter genes were utilized; a simple promoter containing three tandem copies of a consensus hormone response element (3XHRE) and a complex prostate specific promoter, PSA luciferase, which contains an upstream androgen response enhancer region and the PSA proximal promoter.

Examination of flutamide action in CV-1 cells on both simple and complex promoters illustrated an agonist effect at a higher concentration (Fig 1 C and D). Flutamide agonist action was not observed on either the simple or complex promoter in PC3 cells (Fig 1A & B). Flutamide agonist action has been previously observed, and in fact antiandrogen withdrawal syndrome has been attributed in part to the potential agonist action of flutamide (Yeh et al 1997). In vitro, flutamide agonist activity was demonstrated in LNCaP cells which express a mutated AR (T877A) which allows for promiscuous activation of the receptor by other steroid hormones, as well as agonist activation by flutamide.

To fully explore differences in antiandrogen action we also proposed the use of the cell lines, PC-3 ^{hAR} and PC-3$^{LNCaP\ AR}$. The PC-3$^{LNCaP\ AR}$ was generated in our laboratory by stable transfection of a mutated AR (T877A) to mimic the LNCaP mutated receptor. The PC-3^{AR} cell line was a kind gift from Dr. Burnstein, University of Miami School of Medicine, Fl., US and contains a stably transfected wild type AR. Published data, as well as evidence produced in our laboratory, indicated the androgen responsiveness of these cell lines (Fig 2). In the case of the PC3–hAR cell line, specific androgen binding and immunoblotting confirmed the expression of AR protein, and androgen transactivation of the MMTV luciferase reporter was also observed (Heisler *et al.,* 1997). Transfection studies of PC3- LNCaP AR have shown the ability of the LNCaP AR to induce a luciferase

reporter system (see Fig. 2). Androgen stimulation of both PC3-hAR and PC3-$^{LNCaP\ AR}$ cell lines illustrated altered AR expression on DHT stimulation, in agreement with previous studies (Dai *et al.*1996). In transfection analysis with these PC3 cell lines no differential effect of bicalutamide or flutamide was observed (Data not shown). It could be considered that expression of AR is not sufficient to highlight any potential differences.

In conclusion the numerous promoters utilized and various cell lines utilized did not accentuate any potential differences between flutamide and bicalutamide. Due to lack of observed effects of the antiandrogens in vitro, we proceeded with our in vivo studies. It was anticipated that AR mutations generated by exposure to both flutamide and bicalutamide may highlight potentially important amino acids involved in the differential actions of the antiandrogens and thus provide a more successful mean of elucidating the mechanisms of action of these antiandrogens.

Task 2 To examine PC-3 and VCaP xenograft mouse models treated with different therapy regimes can result in agent-specific AR variants.

Rationale:

The primary hypothesis of this proposal is that different treatment regimes can select for specific variant ARs that offer a growth advantage to PCa tumors. TRAMP mice have indicated that mutations found in castrated mice segregate to the N-terminal region of AR but those identified in intact mice were located to the LBD suggesting hormonal influence on the type of AR mutations (Han *et al.,* 2001). Mutation analysis of bicalutamide treated patients has also highlighted mutations that were not observed in flutamide–treated patients (Haapala *et al.,* 2001). Recently tissue removed from a patient treated with bicalutamide only exhibited tumor growth in SCID mice in the presence of bicalutamide. Sequence analysis identified the mutation at W741C (Yoshida T *et al.,* 2005).

Experimental Strategy:

It was proposed to generate mouse xenograft models by implantation of four cell lines PC-3 ^{hAR}, PC3-$^{LNCaP\text{-}AR}$, VCaP and PC-3 into SCID mice. It was expected that hormonal manipulation of these mice, by castration and antiandrogen treatment, would provide different selection pressures for variant ARs. Xenograft models and are an invaluable tool have been extensively utilized in the field of prostate cancer research. They offer the

advantage of allowing cell lines to grow in an environment that replicates the in vivo more closely than the in vitro situation.

To analyze androgen receptor variation from intact and castrate xenografts, tumors were excised once reaching 2.0cc, RNA and/or DNA extracted (RNeasy, Qiagen) and RNA reverse transcribed in preparation for amplification with specific primers that span the entire coding region of AR (table 1). All PCR products were verified by gel analysis and purified using QIAquick PCR purification kit (Qiagen), prior to cloning into pGEM T-easy vectors (Promega) using optimized product to vector ratios. A total of 10 AR cDNA's were subcloned and sequenced per tumor. To remove possibility of variation due to reverse transcriptase and/or PCR, all tumors were analyzed twice. Thus 20 AR cDNA's were analyzed per tumor. DNA sequenced from VCaP xenograft tumors were aligned to the AR reference sequence NM_000044 using Sequencher software (version 4.1, Gene Codes, Ann Arbor Mi). A schematic of experimental approach in highlighted in (Fig 6a), with on exception, we isolated RNA and DNA from intact and castrate xenograft tumors only.

A distinct disadvantage of the xenograft model is that it requires the use of prostate cell lines that have already achieved the capacity to proliferate in the absence of androgen, a key feature of recurrent prostate cancer. The PC3 parental cell line does not express an active AR and will proliferate in the absence of androgen in vitro. The VCaP cell line was isolated from a prostate cancer vertebral metastasis of a flutamide treated patient. VCaPs express AR and have shown androgen sensitive growth both *in vivo* and *in vitro*. Interestingly the VCaP cell line was isolated from a patient previously exposed to flutamide treatment. Thus this cell line may not provide us with further information on the selective pressure of the antiandrogens. (Korenchuk et al., 2001). Since we are interested in the mechanisms of androgen resistance via the selective pressures of the antiandrogens and given the limitations of the prostate cell lines used, we considered it prudent to test our xenograft models with castration prior to hormonal manipulation with specific antiandrogens. Previous studies in TRAMP mice have indicated that castration can effectively generate hormone specific AR variants (Han *et al.,* 2001) Results using xenograft models are discussed in detail below, briefly however, many of the cell lines lost ability to response to androgen withdrawal when grafted into SCID mice (Fig 3). As a consequence we extended our study to include a second model system previously generated in our laboratory (Albertelli et al 2006), the humanized AR mouse crossed to TRAMP mice (h/mARx TRAMP).

To study the influence of AR polymorphisms on prostate cancer initiation and progression, we generated mouse strains carrying human AR N-terminal sequences in place of the mouse N-terminal. Since the N- terminal region of the human AR is most divergent from mouse AR, this strategy generated a strain of mice with a 'humanized' AR (hAR). Generation of humanized mouse was described in detail, (Albertelli et al 2006). Crossing the h/mARmouse to the prostate cancer mouse model, TRAMP, resulted in mice that show similar characteristics to human PCa progression with PIN lesions arising at 12 wks of age (Albertelli et al 2008). This model offers the unique opportunity to investigate the human AR in an in vivo setting, but also in a homogenous genetic background. Consequently we proposed to test our hypothesis that prostate treatment can provide a selection pressure on production of AR mutations using these h/mARx TRAMP mice. The proposed experimental strategy is highlighted in Fig. 6

The h/mAR x TRAMP mice began treatment with antiandrogen (Flutamide 25 mg/Kg and Casodex 50 mg/Kg) at 12 wks, or at the time of first positive tumor palpation. Published data indicates that at 12 wks TRAMP mice exhibit PIN, considered to be a precancerous lesion of the prostate. MRI as well as weekly assessment for palpable tumors assessed tumor initiation and abdominal palpation was found to be as effective as MRI. Analysis of tumor growth and treatment length differences between flutamide and casodex was observed. Once mice become moribund, necropsy was carried out, tumors harvested and prepared for AR sequence analysis as outlined previously for xenografted SCID mice.

The treatment time points chosen (12 weeks of age or at the time of first palpable tumor) represent early versus later treatment. At 12 weeks of age multiple studies have confirmed the presence of PIN, sometimes considered the precursor of oncogenesis. Treatment beginning at the time of first palpable tumor is similar to treatment of patients with local or advanced local disease. Most studies involving TRAMP mice study androgen withdrawal, via castration (Johnson et al 2005, Wikström et al 2005 Han et al 2000), rather than the blockage of hormone action via antagonistic action of the antiandrogens. Castrated mice were included in this study as a means of comparison to other studies and to highlight any potential differences between androgen ablation techniques.

Analysis of AR mutation was carried out using similar protocol as that used for VCaP xenogrfated tumors. A schematic of experimental approach in highlighted in (Fig 6a). Different primers and PCR parameters were utilized (Table 1) due to nucleotide

differences between human and mouse sequence. To analysis AR sequence changes all AR sequences isolated from humanized AR mouse where compared with recombinant DNA fragment encompassing most of human Ar (amino acid 31- 484) and mouse 5' regulatory sequence and exons two to eight (Albertelli et al 2006.). In order to compare mutations found in our h/mARXTRAMP mice to those mutations previously identified in the Androgen receptor database we converted amino acid numbers to match the nomenclature used in the AR reference sequence NM_000044 (920 aa, 23 Q and 23 G).

A

Primer Set	Primer Sequence (5'→3')	PCR profile	Fragment Size (bp)
1	F-cggggtaagggaagtaggtg †	94°C-45 sec, 55°C-30 sec, 68°C-90 sec.	658
	R-ctgccttcggatactgcttc		
2	F-caactccttcagcaacagca	94°C-45 sec, 55°C-30 sec, 68°C-90 sec,	777
	R-cacacggtccatacaactgg		
3	Ftcatcctggcacactctcttcaca	94°C-45 sec, 60°C-30 sec, 68°C-90 sec,	281
	Rggggcccatttcgcttttgacaca		
4	F-gtgagcagagtgccctatc	94°C-45 sec, 55°C-30 sec, 68°C-90 sec,	759
	R-tcctggagttgacattggtg		
5	F-gaccagatggctgtcattca	94°C-45 sec, 55°C-30 sec, 68°C-90 sec,	652
	R-ggaaattccccaaggcactg††		
6	F-tcggtggaagctacagacaa †	94°C-45 sec, 57°C-30 sec, 68°C-90 sec	755
	R-ccgacactgccttacacaac		
7	F-ttcgaccatttctgacaacg	94°C-45 sec, 57°C-30 sec, 68°C-90 sec.	965
	R-ttggtcaaaaggaggcattt *		
8	F-agtgtgggtaccctggtggag	94°C-45 sec, 55°C-30 sec, 68°C-90 sec.	831
	R-ttgtgcatgcggtactcatt		
9	F-caacttgcatgtggatgacc	94°C-45 sec 55°C-30 sec, 68°C-90 sec	631
	R-tctggaaagggaacaaggtg ††		

B

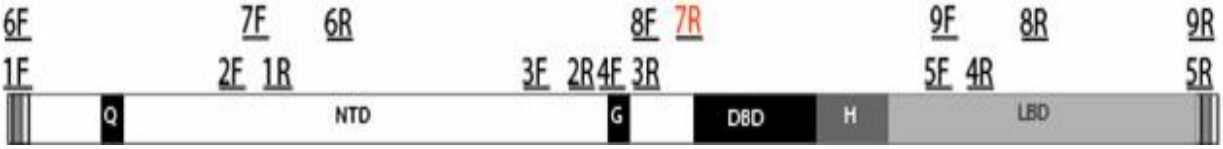

Table 1,
A .Details of primers used for amplification of the Androgen receptor. †, indicates that primers is located in the 5" UTR, †† in the 3'UTR and * indicates that this primer is found in the AR intronic sequence; mouse chromosome X reference assemble, NC_00086. All PCR profiles included an initial 5 min denaturing step at 94°C and a final 10 min elongation step at 68°C.
B Location of primers on AR. Primers 1-5 were utilized form amplification of AR from xenograft prostate tumors while primers set 6-9 were utilized to amplify humanized AR from h21Q x TRAMP mice. Primer 7R highlighted in red indicates primer location within the intron

Results/Discussion

PC3, PC-3 ^{hAR}, PC3-$^{LNCaP\text{-}AR}$ xenografted tumors

As was highlighted in previous reports, early experimental results indicated that the xenograft model system utilizing the PC3, PC3^{-hAR}, PC3$^{-LNCaPAR}$ cell lines may be inappropriate for investigating the potential selection pressures of the antiandrogens *in vivo*. These cell lines once injected into nude mice failed to show hormonal response to castration. No response to androgen ablation was seen when tumor growth was measured (Fig 3) despite the fact that these cells lines were shown to be androgen responsive in transfection analysis in vitro (Fig 2). This result was expected for PC3 cell lines as they do not express an active AR and already have achieved the capacity to proliferate in the absence of androgen. To explain the lack of hormonal response in vivo it may be considered that stimulation of an alternative androgen independent growth signaling pathway in the xenografted tumour cells may override any potential influence the stably transfected AR may have on proliferation. It may also be suggested that the expression of AR in PC3 stably transfected cell lines may be too low to exhibit potent androgen responsiveness in vivo.

Data obtained suggested that it is impossible to assess selective pressure of the hormonal environment on the AR in these cell lines, consequently further analysis of these xenografted mice was halted. Therapeutic failure and development of androgen independent disease despite the presence of the AR is a major problem in treatment of prostate cancer and it appears that PC3$^{-h/mAR}$and PC3^{-LNCaP} xenografts may be an illustration of this late stage of prostate cancer.

VCaP xenografted tumors

The VCaP cell line did show hormonal response *in vivo* (See fig 4a) with androgen withdrawal initially slowing xenograft growth and then exhibiting androgen independent growth. Using immuncytochemical techniques AR protein was localized to the nucleus in tumors isolated from intact SCID mice while those isolated from castrate mice exhibited some cytosolic staining. Positive staining within the nucleus was also seen in castrate mice. To assess if relative amount of AR protein was altered depending on hormonal status AR immunoblotting was carried out and normalized to βtublin levels. No alterations in AR levels were observed (Fig 4 B3).

Since hormonal responsiveness of VCaP cells lines was illustrated we analyzed VCaP androgen receptor variation from intact and castrate xenografted tumors. A total of 190 AR sequence differences were identified in VCaP xenogrfated tumors, a total of 92 mutations were identified from tumors removed from intact mice and 98 mutations were identified from castrate mice. For complete details of mutation type see table 2, the majority of mutations were missense mutations, (66% in intact versus 60% in castrate). A mutation rate of 3.6 mutations per 10,000 base pairs sequenced was calculated for both intact and castrate tumors, with each tumor examined having on average 24 mutations per tumor.

	Intact Tumors	Castrate Tumors
# Tumors Analyzed	4	4
Total number of mutations	92	97
Missense	61	59
Deletion-Frameshift	10	15
Insertion - Frameshift	0	1
Nonsense	2	4
Silents	15	16
Codon insertion	3	0
Codon Deletion	1	3

Table 2-Characteristics of all AR mutations identified in VCaP xenogrfated tumors

A major problem with the sequencing strategy utilized in this study is the unequivocal identification of true mutations. Two independent reverse transcriptase reactions were carried out in an effort to control for reverse transcriptase error (1 per 15,000 base pair, as reported by the manufacturer), and a proofreading DNA polymerase (error rate of 1.58 per 100,000 base pair) was utilized to amplify AR fragments that covered the entire AR coding sequence. It is possible however that the inherent error rates of enzymes may contribute to the high mutation rate.

Since sequence changes that occur more than once have a higher probability of being true mutations it was decided to classify mutations according to their pattern of recurrence. It

was observed that mutations fell into four categories; (1)-mutations that were duplicated between RT reactions, (2) sequence changes that occurred in more than one tumor and that resulted in a the same codon changes in all tumors examined, (3)-Nucleotide change at the same codon site in multiple tumors that resulted in different protein change at that codon number in each tumor, (4)- mutations that occurred greater than 10% of clones analyzed. As detailed in the experimental protocol we sequenced at least 10 clones per AR fragment, thus genetic alterations in this category were found in more than one clone.

Mutations in AR isolated from VCaP xenogrfated tumors that fell into the four identified categories are illustrated in fig 4. Only one mutation was duplicated in two independent RT reactions, Q58L resulting in a leucine placed immediately upstream of the polyglutamine tract (Q Tract). This has been previously reported to be identified in two infertile men (Lund et al 2003). It can be postulated that Q58L could change the conformation of the polyglutamide tract thereby influencing the binding of co-activators and co-repressors. Interestingly, a second mutation in this area was identified in more than one tumor, L57Q. This mutation has the opposing effect of lengthening the Q tract and has been associated with both prostate and liver cancer. Glutamine 525, mutated in this study, has previously been shown to be mutated in recurring prostate cancer patients that were treated with a combination of castration and the cytotoxic drug estramustine phosphate. In fact a clustering of mutations was observed in this region that was not observed in patients with castration alone, suggesting a distinct selective pressure (Hyytinen et al, 2002).

Many of the codons altered in this study have been shown to be associated with androgen insensitivity syndrome (Turek-Plewa et al, 2006, Kohler, et al, 2005, Komori et al, 1997, Brown et al 1992, Saunders et al 1992). Androgen insensitivity syndrome is often associated with the decreased androgen receptor activity. The identification mutations in our xenografted tumors which have previously been associated with this syndrome appear contradictory. Mutations of AR in prostate cancer are mostly considered to be gain of function (He et al 2006). However, mutations must be considered in terms of the altered hormonal context of prostate cancer. Somatic mutations associated with loss of AR function in normal physiology may be associated with AR gain of function within a prostate cancer tumor.

It has been previously reported that AR mutations can cluster in different functional regions of the AR depending on the hormonal status. Han et al (2001) observed that

mutations isolated from castrate mice localized to transactivation domain while those identified from intact mice were found in ligand binding domain. Hormonal dependent clustering along AR functional regions was not observed in our mice. As shown in fig 4c mutations from intact and castrate (underlined mutations) mice were localized along the entire coding region. Interestingly there were few mutations that occurred in both intact and castrate mice (highlighted with black circle, fig 4c). This is suggestive of distinct hormonal selective pressure on development of AR genetic changes.

h/mARxTRAMP Mice

With the recognition that the xenograft mouse model has certain limitations we decided to utilize an alternative experimental strategy while maintaining the overall proposal objective. We focused out attention on an alternative mouse model of prostate cancer that was generated in our laboratory, the humanized androgen receptor mouse model of prostate cancer- h/mARX TRAMP.

A principle task was to establish that h/mAR x TRAMP mice was hormonally responsive to antiandrogen treatment. Certainly much work has been done on the TRAMP mouse however most studies have utilized orchidectomy rather than AR antagonism to generate androgen independent state (Wikström et al 2005, Johnson et al 2005, Huss et al 2007 Albertelli et al 2008). Therefore we initially studied a subset of h/mARxTRAMP mice treated with bicalutamide and flutamide at 12 weeks of age for a period of 4 weeks. To examine hormonal influence we measured seminal vesicle weight at the end of the androgen ablation treatment. Seminal vesicles are androgen responsive and weight change is often used as a bioassay for androgen (Chai et al 1956). As shown in Fig 5 a seminal vesicle in androgen ablated mice was significantly decreased when compared to hormonally intact mice ($P = 0.0003$ ANOVA). Testosterone analysis also showed a decrease in testosterone levels in all treatment groups when compared to intact mice. A decrease in testosterone levels in castrate mice is expected, the effect of the antiandrogens is not so clear. In humans, antiandrogen treatment raised testosterone levels (Tyrell et al 2006). In rats bicalutamide (25mg/kg) had no significant effect on serum testosterone or LH. Similar results were observed in dogs treated high dose of bicalutamide 100mh/kg over a 28 day period. However flutamide was shown to elevate testosterone even at a lower dose of 5 mg/Kg (Furrr and Tucker, 1996). The endocrine difference between the antiandrogens was explained by the lack of peripheral selectivity of flutamide in rats. Differences between human and rodent endocrine response are difficult

to explain. It must be noted however that human serum testosterone concentration has been shown to plateaus and remain within the normal range for most patients treated with bicalutamide (Verhelst et al 1994). Measurement of testosterone in mice is notoriously difficult and clarification of the endocrine response of testosterone production to bicalutamide and flutamide treatment levels would require greater number of mice with blood sampling taken at more than one time point over an extended period.

To aid clarification of the hormonal response within the prostate of h/mARxTRAMP mice, we localized the AR using immunocytochemistry. In the prostates of mice treated with antiandrogens, AR positive staining was localized to the nucleus of epithelium lining the prostate ducts in untreated control mice. Some staining was also observed in the surrounding stroma (Fig 5 B). A similar pattern of staining was observed in both flutamide and bicalutamide treated mice in accordance with previous reports (Waller et al 2000). AR staining in castrated mice appears cytosolic. Decreased seminal vesicle weight and altered AR localization on treatment androgen ablation techniques are appropriate responses to hormonal therapy. Consequently h/mARxTRAMP mouse is an appropriate model to test our hypothesis: agent specific AR variants are generated during treatment of prostate cancer and can aid tumor progression.

h/mARxTRAMP mutations

A total of 994 genetic alterations were identified in h/mARXTRAMP mice. This excludes Q tract and G tract deletions. Complete analysis of mutation type is seen in table 3. A mutation frequency of 4.0 /10000 base pairs examined was calculated, very similar to that found in our xenograft mutation analysis (3.8 changes/10000 base pairs analyzed). The high frequency of mutations may be explained by the type of tumor analyzed. All tumors were end stage and removed when mouse became moribund.

	Intact	Castrated	Bicalutamide	Flutamide
# tumors analyzed	10	10	9	9
Number of Mutation	**217**	**253**	**241**	**283**
Number of missense	121	141	114	162
Number of nonsense	16	16	19	29
Number of Deletions-frameshift	0	14	2	0
Number of insertions-frameshift	18	0	14	15
Number of Silents / tumor	57	72	75	68

Table 3 Characteristics of mutations (excluding deletions in Q and G tract) identified in h/mARXTRAMP mice with different treatment regimes, Intact (no treatment), Castrate mice, Bicalutamide (50mg/kg) and Flutamide (25mg/kg).

Precautions regarding experimental protocols (proofreading polymerase, duplicate RT reactions, and analysis of multiple subclones) were taken but despite this, the identification of true mutations is extremely difficult. In an effort to obtain an insight into the minimum error rate in the sequencing process AR cDNA was sequenced from h/mAR testis. The testis of this transgenic mouse does not contain the SV40 T antigen and few mutations would consequently be expressed. Analysis of AR sequences isolated from 17 mice indicated that there was a mutation rate of 2.2 mutations/10000 base pairs (personal communication Dr M Albertelli). These mutations are presumably the result of errors introduced during the experimental protocol. This suggests that possibly 50% of mutations identified in this study are not true mutations.

In an effort to increase stringency of the mutation analysis, it was decided to classify mutations according to their pattern of recurrence, as previously discussed for VCaP xenograft mutation analysis. Sequence changes that occur more than once have a higher probability of being true mutations. Mutations duplicated between repeat RT's, those found in greater than 10% of sequences analyzed per tumor and those found to recur in multiple tumors were identified in can be found listed in the appendix Tables 1-3. For a schematic representation of the altered amino acids of the AR protein see fig 6 b. All alterations occurred in more than one tumor and those mutations highlighted in red indicate those nucleotide changes that result in the same protein change in all tumors examined. Tilley et al 1996 suggested that mutation of the transactivation domain was a high frequency event. In our study 49% of recurring mutations occur in this domain which is in agreement with Tilley et al. 1996. Taplin et al 2003 analyzed AR transactivation sequences yet they did not find any mutations in this region. Discrepancy between these results may be explained by the type of tissue used. Tilley et al analyzed biologically aggressive tumors where 40% of the cohort studied progressed rapidly to androgen independent disease. Taplin et al 2003 analyzed bone marrow aspirate and biopsies. A major advantage of the humanized mouse model of prostate cancer is that potential heterogeneity caused by patient selection can be avoided.

As indicated by the AR schematic, amino acids are mutated throughout the AR coding region although some clustering can be seen in both Q and G tracts of the N terminal. Hot spots for mutations usually highlight areas of functional importance and clustering of altered amino acids is also observed in DBD and LBD regions of the AR. Both DBD and

LBD regions are highly conserved among the steroid receptor family and are essential for correct function of the AR. Previous reports of AR mutations in the LBD region have reported to broaden ligand specificity. Examples of which include the LNCaP T877A mutation which allows flutamide to act as an agonist and W741C which is associated with bicalutamide agonist activity (Tan et al 1997, Haapala et al 2001). Of the 48 codons found to be altered in the LBD, 33 have been previously been shown to be mutated in either androgen insensitivity syndromes and/or prostate cancer (Androgen receptor database, http://androgendb.mcgill.ca/). In the DBD region (aa 538-628) 19 amino acids were altered in this study, of which only 3 amino acids have been previously been reported C560, A597, R609. C560 is highly conserved amino acid of DNA zinc finger region of the steroid receptors. The C560W mutation reported by Zoppi et al 1992 showed no transcriptional activity in transfection assays due to decrease ability to bind to DNA. Although our amino acid change are not the same (C560F and C560S), it can be postulated that any change of such a highly conserved amino acid would result in a similar effect in DNA binding and transcriptional activity. We also noted an amino acid substitution in A596 which is located in the second zinc finger region called the D-box. The D box comprises of 5 residues that along with the LBD is involved with receptor dimerisation. Alteration of this amino acid to theronine resulted in a receptor that had reduced transactivation ability in vitro (less than 30% of wild type) but could regain function at higher ligand concentrations (Giwereman et al 2000).

A major hypothesis of our research proposal was that treatment specific genetic alterations occur during prostate cancer progression. The venn diagram analysis of recurring mutations indicate that few mutations belong solely to one hormonal group, in fact 20 mutations were shared between intact and castrate mice and 26 between the antiandrogens flutamide and bicalutamide. Interestingly a high number of mutations were found in flutamide treated group (19) with only a very small number associated with casodex only treated mice (3). This may hint at differential mechanisms of tumor progression in bicalutamide treated mice when compared to flutamide treated mice. The partial agonist effect of flutamide may also contribute to the difference number of mutations. Due to the common use of combined androgen ablation therapy (medical castration with antiandrogen treatment) it is difficult to compare this sequence data obtained in this study to human studies. The hARxTRAMP mouse offers the distinct advantage of examining endocrine therapies that would not be permissible in the human population.

To increase the stringency of our analysis we focused on mutations that were found to occur in more than one tumor and resulted in the same codon change in all tumors examined. We also included duplicated mutations (mutations repeated between two RTs) and those mutations that occurred in more than 10% of sequence analysis. Fig 7 illustrates a localization of these amino acid substitutions. The same clustering pattern that was seen in the less stringent cohort of mutations is observed. Mutations are classified according to hormonal group. As was evident from previous venn diagram analysis few mutations occur in a single group. Interestingly however there are fewer recurring mutations in mice that received no treatment (Highlighted in blue). This supports the hypothesis that treatment supplies a selective pressure on the generation of AR mutations.

The primary objective of this proposal was the identification of AR variants that could contribute to the development of androgen independent disease; however careful analysis of tumor progression in these mice also provided an insight into differential actions of specific hormonal therapy in terms of survival and tumor detection. Antiandrogen treatment at the sign of first detectable tumor mimics prostate cancer patient treatment, while treatment beginning at 12 weeks provides a model to test the influence of early treatment on mouse survival. By following tumor progression by weekly abdominal palpation of our h/mAR xTRAMP mice we have been able to analysis time to tumor progression and survival in the presence of the antiandrogen bicalutamide and flutamide. This information provides an insight into mechanisms of androgen independent disease progression in treated subjects as well as illustrates the relevance of h/mAR x TRAMP mouse as a model of prostate cancer.

Survival Studies

Androgen withdrawal, via medical or surgical means is considered the main form of hormone therapy for advanced local disease. In this study we investigated whether antiandrogen treatment would increase survival time (measured form birth to death). Global analysis of data shows a statistically significant difference between intact and antiandrogen treated mice (P=0.0026).

When comparing early versus later treatment with antiandrogen Kaplan Meier analysis (Fig 8 A) highlighted a significant difference in survival between untreated mice and those treated upon tumor detection with flutamide and bicalutamide (P= 0.0023 and P=0.0015

respectively). Androgen is required for both proliferation and differentiation with the prostate and antiandrogens competitively bind AR and prevent downstream signaling. Androgen sensitive cells within the antiandorgen treated prostate tumor will apoptosis and initially decrease tumor proliferation and influence overall survival. In contrast early treatment at 12 weeks of age with either flutamide or casodex, did not offer a survival advantage relative to intact mice (P= 0.58, P=0.043, respectively). Johnson et al proposes that a subset of androgen independent cells preexist within the heterogeneous tumor and the antiandrogen treatment will allow for uninhibited growth of these cells. Early exposure of the tumor to antiandrogens allows for expansion androgen independent tumor cells and thus decreases overall survival. Comparing bicalutamide with intact mice shows a similar slope in graph. Interestingly flutamide treatment shows a slightly different slope, similar to castrated mice, where some mice appear to benefit from this antiandrogen treatment. The effect of castration will be discussed in detail later but one can suggest that the slight difference in slope may be due to the known agonist effect of flutamide. More mice would be required to investigate further.

Of interest is the fact that a significant difference in survival was observed between non treated and castrated mice. However as indicated in figure 8 data obtained from castrate mice is somewhat complicated, some mice disease progresses rapidly and while other survive for an extended period of time in the absence of testosterone. This trend has been observed in previous reports Wikstrom et al illustrated that over 55% of long term castrated mice (castrated at 24 weeks of age with no palpable tumor) relapsed with local and/or metastatic disease while 45% were cancer free at age one year. Although ratio of cancer free to relapsed mice was different (20% cancer free versus 80% with poorly differentiated tumors on necropsy at 24 weeks), Gingrich et al 1997, also highlighted the dual effects of androgen withdrawal.

This survival data presented highlights differences between complete androgen withdrawal and androgen receptor blockage at the same time point. While the exact mechanisms remain elusive it may be suggested that AR remains active even in the castrated animal. Testosterone and DHT have been measured in recurrent prostate cancer specimens at levels high enough to activate the AR (Titus et al 2005). Ligand independent activation of the non-liganded AR may also be proposed. Binding of the antiandrogens is known to result in a conformational change which can alter the binding surface for available co-

regulators, thus changing AR downstream signaling mechanisms which may contribute to the difference in overall survival pattern evident in this study.

Tumor Detection

Prostate cancer in men is a slowly progressing disease; achieving even a slight delay in development and progression of prostate cancer could substantially reduce the clinical impact of the disease. Similar to the human disease the TRAMP mouse model prostate cancer progresses through well defined stages (Kaplan-Lefko et al 2003). We investigated if antiandrogen treatment influences initial tumor detection the h/mAR X TRAMP mice.

Kaplan-Meier analysis indicated that there was no overall significant difference in initial tumor detection between antiandrogen treated groups. The timing of treatment with antiandrogen does not influence disease detection; there is no difference between early and later treatment with either flutamide or bicalutamide (Fig 8B). Analysis of graphical data, could suggest that mice treated with biclutamide on first palpable tumor show a slight increase in time to detection of tumor. However the median time to tumor detection for bicalutamide treated mice (28.5) falls with the intact groups 95% confidence interval (25.7, 30.7). Castration at 12 weeks has been shown in a previous study to have no effect on tumor detection, although some mice did present with an earlier palpable tumor relative to intact mice (Albertelli et al 2008). While some castrated mice in this study also presented with an earlier palpable tumor, among all castrated and antiandrogen mice there is not a statistically significant difference in time to tumor detection.

Although androgen withdrawal has been an accepted treatment regime since the work of Huggins and Hodges, no definitive treatment strategy has been proposed for prostate cancer. Treatment regimes can involve orchidectomy, medial castration alone or with antiandrogen treatment. Often radiotherapy can involve neoadjuvant as well as adjuvant hormonal therapy. In Europe watchful waiting is often considered the best treatment action. Numerous clinical trials have been carried out to estimate the value of different treatment strategies. The importance of clinical trials will never be replaced but from preliminary studies here with small groups of humanized TRAMP mice evidence suggests that these mice are valuable tools for preclinical evaluation of hormonal and possibly other treatment regimes.

Task 3 To characterize functionally AR molecular variants that may result from antiandrogen treatment of the mouse xenograft models and to test their capacity for tumor promotion.

We identified a 69bp insert from our VCaP xenografts (see fig 4). This AR mutation was of particular interest as it was also found in 5 of 8 antiandrogen treated human prostate cancer patients that were sequenced in another project of in our laboratory. This insertion results in an androgen receptor with a 23 extra amino acids between the two zinc fingers of the DNA binding domain and is the result of aberrant splicing of intron 2. This insert has also been reported in a family with partial androgen insensitivity. Although *in vitro* analysis highlighted defective DNA binding and transcription (Bruggenwirth et al,1997) we considered that presence of this mutated androgen receptor within a cancer cell may influence AR protein–protein interactions and potentially influence AR repression. Previously it has been shown that activated AR can decrease TPA stimulated NFkB transactivation (Aarnisalo et al 1999). To examine potential AR repression we examined the effect of the 69bp insert AR on NFkB reporter activity in the presence of TPA and testosterone. As is evident in Fig 9, the mutated AR was unable to repress NFkB activation. Recently published data indicates that this splicing variant has impaired nuclear entry and that cellular localization is predominantly cytoplasmic (Jagla et al 2007). This paper also illustrated that this AR splice variant stimulated NFkB transcriptional activity. Difference in NFkB action in the presence of AR has previously been noted and maybe the consequence of cell type utilized. In our experiments we utilized CV-1 cells where as in Jagla et al 2007 LNCaP cells were utilized. Further study is required for complete analysis. Utilization of xenograft model above had one distinct limitation. VCaP cells are isolated form vertebral metastasis, as a consequence can be considered to be terminally differentiated. It could be proposed that the mutations identified may be indicative of types of mutations generation during tumorigenesis. As previously explained we also focused on the m/h x TRAMP model of prostate cancer and identified many novel mutations. Time constraints did not allow for complete functional analysis of all mutations found in this study. We concentrated on the mutations highlighted below and preliminary transfection analysis was carried out. Results obtained to date are indicated in table 4. It was observed that M524Tshiws high activity in both CV-1 Cells and PC3 cells and on immunoblotting this AR variant migrates at a higher molecular weight. This may be suggestive of altered protein modification but further analysis is required.

The utilization of multiple promoter constructs may highlight promoter specific effects of the mutated AR while different cell types were utilized to illustrate any potential cell specific effects. AR variant R753Q showed differential action on promoters utilized in this study, with low activity exhibited with C'Δ9 and 4HRE2 with normal activity shown on PSA and 3x HRE3 promoter constructs. Further analysis is required and experiments are ongoing in the Robins' laboratory.

Codon Change	Number of tumors with mutation and treatment type	Transfection analysis results
M524T	3 flutamide 3 bicalutamide	normal to high activity in CV-1 and PC3 cells (C'Δ9, PSA, 4X HRE2, 3X HRE3) migrates slower on acrylamide gels
S741F	2 flutamide 2 bicalutamide	Loss of Function no activity in CV-1 or PC3 with R1881
M750I	1 flutamide 1 bicalutamide	low activity in CV-1 normal activity with high concentration (1 μM) R1881 on C'Δ9
W752C	1 flutamide 1 bicalutamide 1 castrate	low activity in CV-1 and PC-3 normal activity with high concentration (1 μM) R1881 on C'Δ9
R753Q	1 flutamide 1 intact	low activity on C'Δ9 and 4X HRE2, normal activity on PSA and 3X HRE3 1 nM R1881 normal activity with high concentration (1 μM) R1881 on C'Δ9
R761G	3 castrate	normal activity in CV-1 and PC3

Table 4 List of mutations functionally analyzed to date. We used transfection analysis with multiple promoter constructs (C'Δ9, 4X HRE2, 3X HRE3, PSA) in a two cell lines, CV-1 and PC3 with differing concentrations of R1881.

Key Research Accomplishments

- Development of VCaP xenograft model that illustrates appropriate hormonal response.
- Identification of novel somatic AR mutations that are found in functionally important regions suggests that they may play a part in influencing prostate cancer disease progression.
- The treatment of the hAR x TRAMP mouse with antiandrogens slows disease progression. Kaplan-Meier analysis indicated that there was no overall significant difference in initial tumor detection between antiandrogen treated groups. The timing of treatment with antiandrogen does not influence disease detection; there is no difference between early and later treatment with either flutamide or bicalutamide.
- In this study we investigated whether antiandrogen treatment would increase survival time (measured form birth to death). Global analysis of data shows a statistically significant difference between intact and antiandrogen treated mice (P=0.0026).
- Few mutations occur in a single hormonal group only. All four groups, intact, castrate, bicalutamide and flutamide having many recurring mutations in common. Interestingly there are fewer recurring mutations in mice that received no treatment (Highlighted in blue, fig 7). This supports the hypothesis that treatment supplies a selective pressure on the generation of AR mutations.
- Early antiandrogen treatment at 12 weeks had no impact on overall survival of h/m AR x TRAMP mice, when compared to non–treated mice. In contrast castration did show significant effect highlighting differences between complete androgen withdrawal and antiandrogen treatment.
- The humanized mouse provides a unique model of prostate cancer that could be utilized to test new drugs

Reportable outcomes

Data generated in this study has been published and presented at local and international meetings. Publication records and abstract details as follows;

Albertelli MA, **O'Mahony, O.A** Brogley, M, Tosoian J, Steinkamp, M, Diagnault, S. Wojno, K., Robins, Glutamine tract length of human androgen receptors affects hormone dependent and independent prostate cancer in mice. Human Mol genetics 2008 Jan 1 17(1) 98-110.

Robins DM, Albertelli MA, **O'Mahony OA.** Androgen receptor variants and prostate cancer in humanized AR mice. J Steroid Biochem Mol Biol. 2008:108(3-5):230-6.

O'Mahony, O.A., Steinkamp, M., Albertelli, M.A., Brogley, M., Butler, T., Gerber, J., Pienta, K., Hofer, M., Robins, D.M. Profiling of androgen receptor mutation in mouse prostate cancer. Manuscript in preparation.

O'Mahony, O.A., Albertelli, M.A., Cadillac, J.M. and Robins, D.M. Effects of Antiandrogens versus Androgen Withdrawal on Prostate Cancer Progression in a Mouse Model. ENDO 2006, Boston, US

Steinkamp, MP, **O'Mahony, OA**, Albertelli, M A., Brogley, M.A, Butler, T, Gerber, J, and Robins, DM. Profiling of Androgen receptor mutations in mouse and human prostate cancer. Endo 2007 Toronto

I was offered a post doctoral research position in Prostate Cancer Research group based in Hammersmith hospital Imperial College London headed by Prof J Waxman. This was a direct consequence of the training I received in Prof D Robins laboratory with the help of this USAMRMC fellowship.

Conclusions;

This study has focused on profiling androgen receptor mutations in xenografts and a humanized mouse model. We identified directly relevant sites in human AR, especially in the n-terminal domain where mouse and human AR are most divergent. We compared mutations in tumors of h/mAR-TRAMP mice following different treatment regimens and compared to non treated mice identified that treatment does confer a selective pressure on AR variant generation. Complete analysis and functional assessment of AR mutations is ongoing. It is proposed that these mutations may lead to greater AR activity under particular conditions (gain-of-function), and potentially highlight sites of interaction with critical cofactors that might themselves serve as novel therapeutic targets to complement androgen ablation.

The utilization of h/m AR X TRAMP mouse offers a distinct advantage over other mouse models, the presence of an AR that is over XX % similar to human amino acid sequence. This study also illustrated for the first time that the m/hAR x TRAMP mouse responds appropriately to antiandrogen treatment. The numbers of mice utilized in this preliminary study was small but early data shows showing similar results with regard to tumor detection and overall survival to those seen in clinical trials with these drugs. While the importance of clinical trials for drug development remains undisputed the m/hAR x TRAMP mouse offers the ability to study the human AR at a molecular and physiological level while also allowing assessment of new treatments in a genetically homogenous environment.

The attainment of androgen resistance remains to be fully elucidated and the fact that no definitive regime for the prevention of prostate cancer progression has been identified compounds the need for further research in the area of prostate cancer therapy. Data obtained in this study allowed for closer examination of antiandrogen function, *in vitro* and *in vivo*, using a novel animal model. Detailed analysis of androgen receptor mutations identified in this study may influence the prognosis and preventative strategy for new patients but also has the potential to highlight novel targets for therapeutic intervention.

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APPENDIX

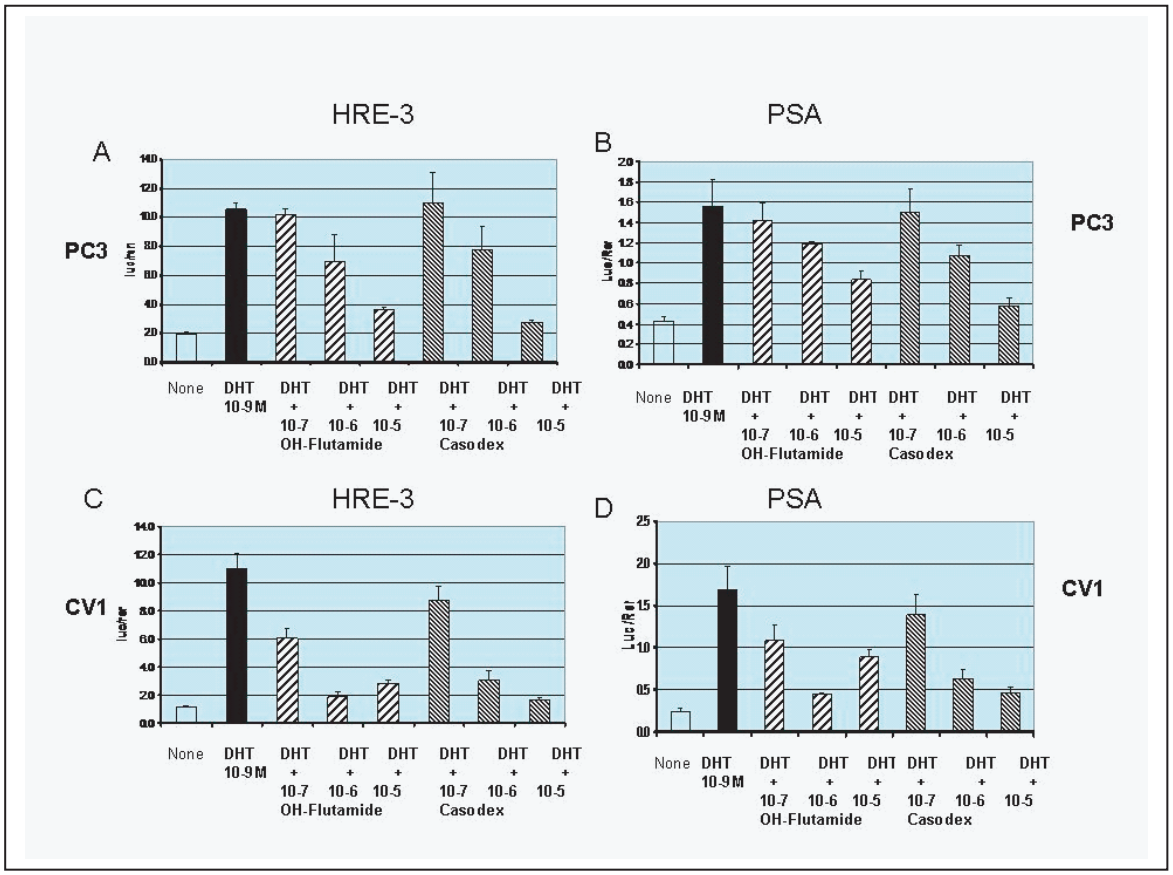


Fig 1 Flutamide agonist action is observed in CV-1 cells on two different promoters at high concentration. No agonist effect was observed in PC- 3. 10^{-5} PC3 or CV1 cells were seeded into 24 well dishes and allowed to adhere for 24 hrs prior to transfection with 4 ng human androgen receptor and 20 ng renilla as a transfection control. Two reporters were utilised 200 ng 3xHRE luciferase reporter or 200ng PSA luciferase reporter.

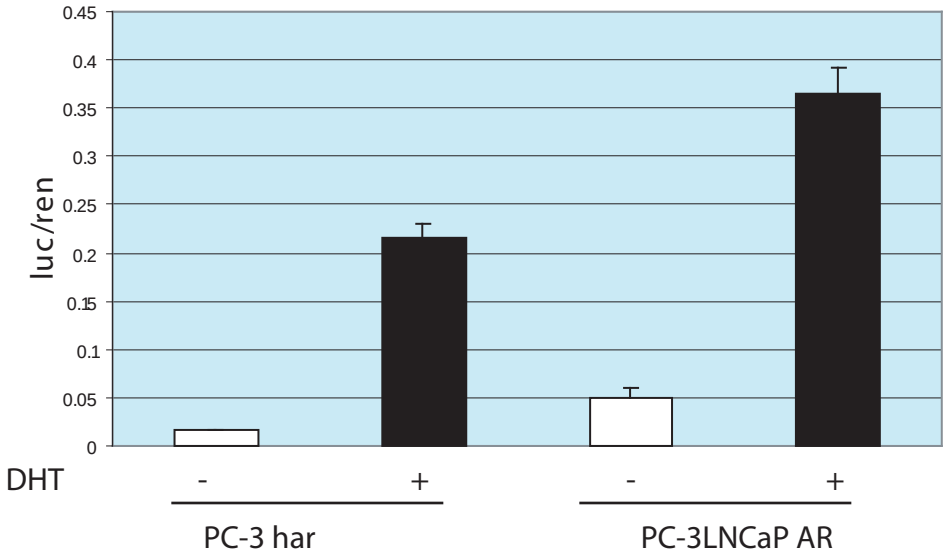


Fig 2 PC3-hAR and PC3-$^{LNCaP\ AR}$ cells were transfected with an androgen specific reporter (C'Δ9 tkta Luciferase, 1µg and CMV- Renilla, 10 ng as transfection control). Twenty-four hours after transfection cells were stimulated for a period of 24hr with $3x10^{-9}$ M DHT and harvested for analysis. Data is expressed as the ratio of firefly luciferase light units over corresponding renilla. (mean± SE).

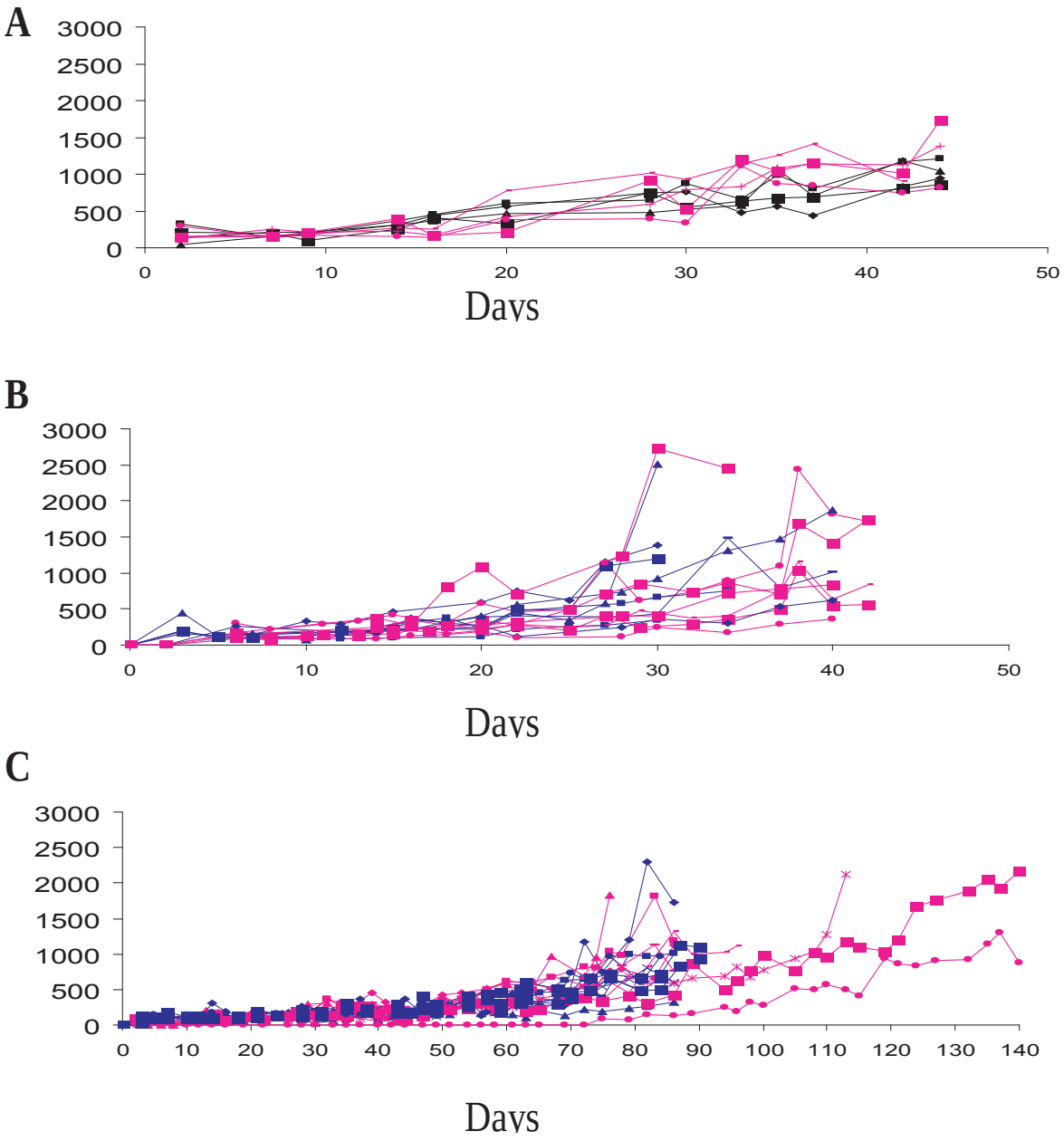


Fig 3 Graphs illustrating tumour volume increase over time in PC3 (**A**)-, -PC3-hAR (**B**), and PC3$^{-\ LNCAP\ AR}$ (**C**) tumour xenografts. 3 million cells in sterile PBS (1:1 ratio with Matrigel) were implanted s.c. into the flank of SCID mice and one week later eight of the mice were castrated. Navy lines represent intact SCID mice and pink lines represent castrated SCID mice.

APPENDIX

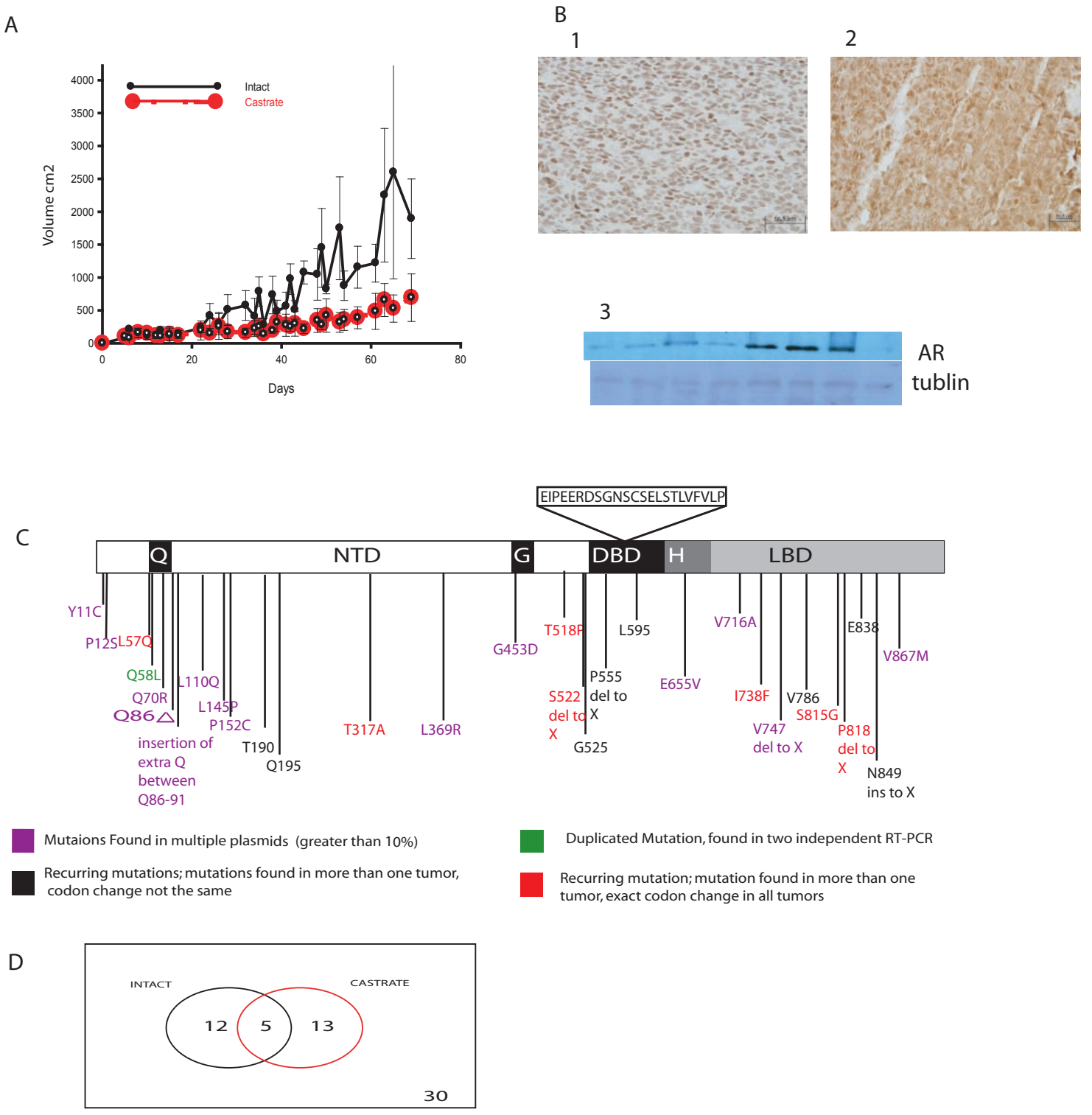
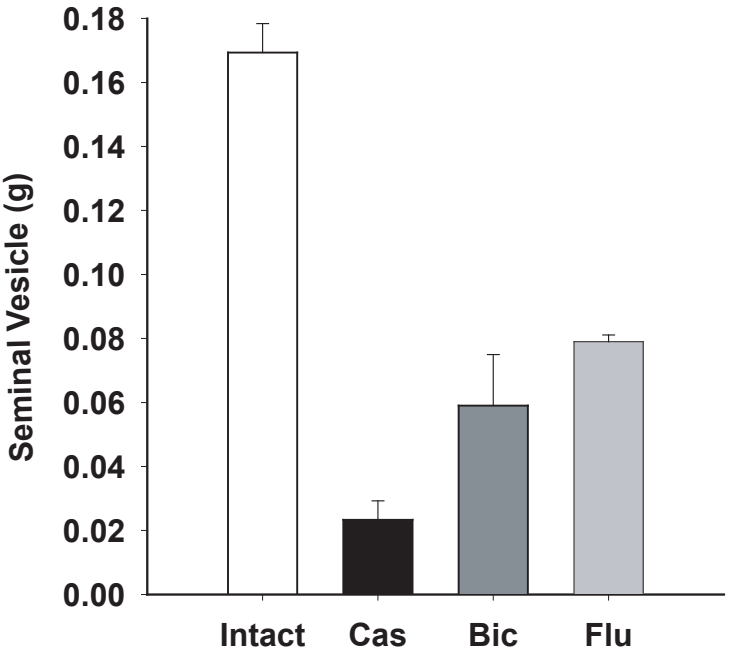


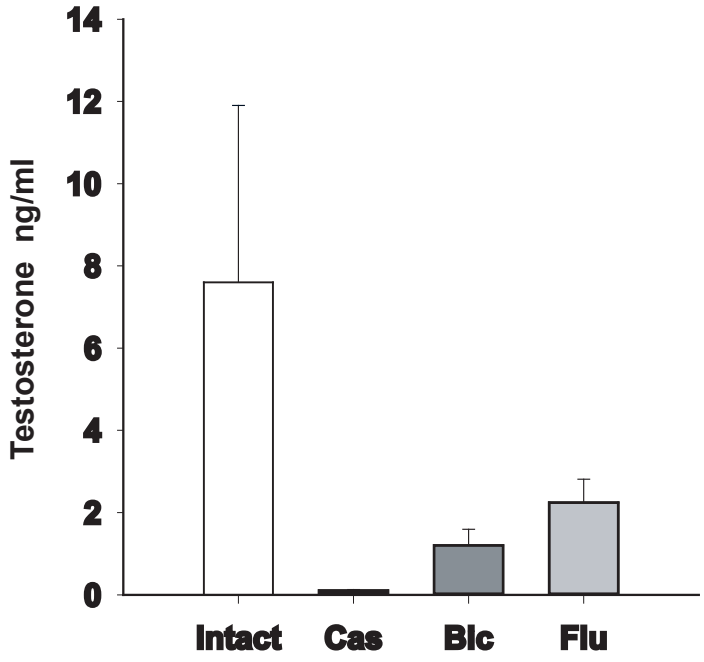
Figure 4 (A) VCaP Xenograft growth curv.e. 3 million cells were implanted into flanks of SCID mice and one week later a subset were castrated. Tumors was measured weekly via calipers and volume calculated via the following formula, (A*(B^2)/2 A= biggest measurement and B shortest measurement.
(B) 1 Immunocytochemistry for AR in tumors excised once volume reached 2 cm2. AR positive staining was evident in both intact (B1) and castrate mice (B2).localisation in castrate mice seems to be presen t in cytosol.
(C) Schematic showing codon changes in AR sequences isolated from VCaP inact anc castrate xenograft tumors. Color codingg explained above.
(D) Venn Diagram representing mutations found and classified according to hormonal status.

APPENDIX

A



B



C

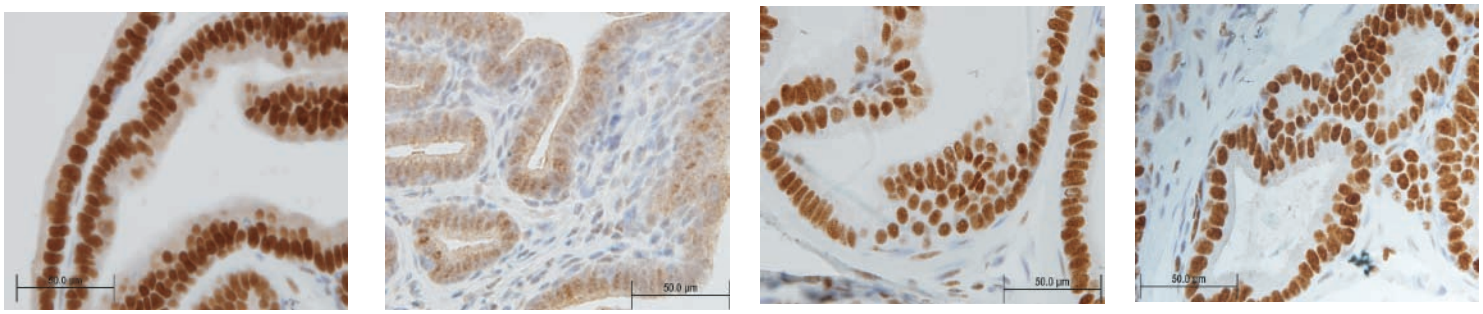


Figure 5 12 week old m/hAR xTRAMp mice (n=12) were castrated or treated with biculatmide or flutamide for a period of 4 weeks.
(A) Seminal vesicles were removed and weighted after 4 weeks treatment.
(B) Testosterone levels were measured in blood taken from the chest cavity from all m/h AR xTRAMP mice
(C) Immunocytochemial localization of AR in prostates removed form treated and untreated m/hAR x TRAMP mice was carried out. AR in untreated mice localized to nucleus of epithelial cells lining the prostatic ducts. A slightly lower insentisty staining was localized to the same region in the antiandrogen treated mice. AR localized to the cytosolic regions of epithelial cells in castrate prostates

APPENDIX

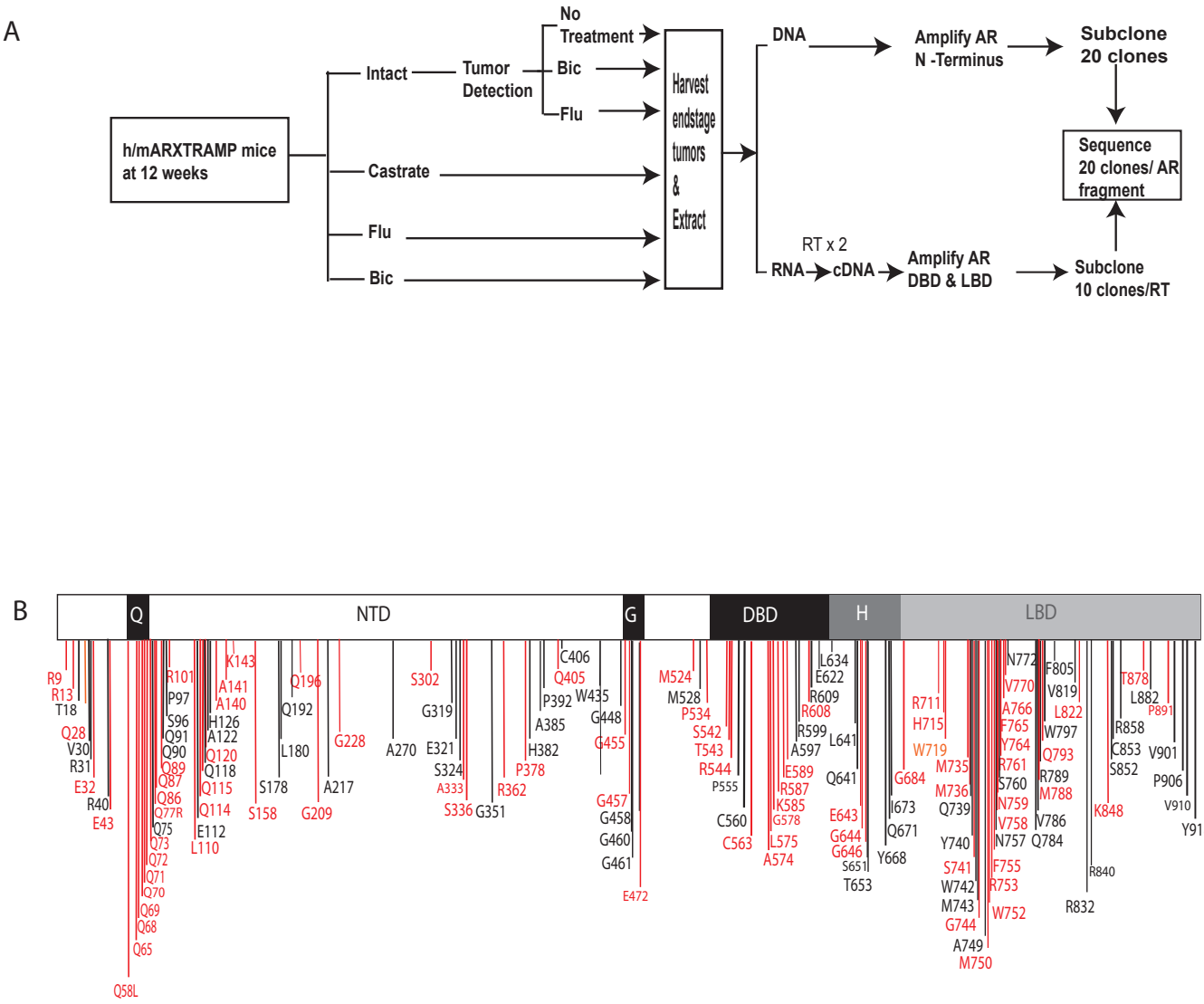
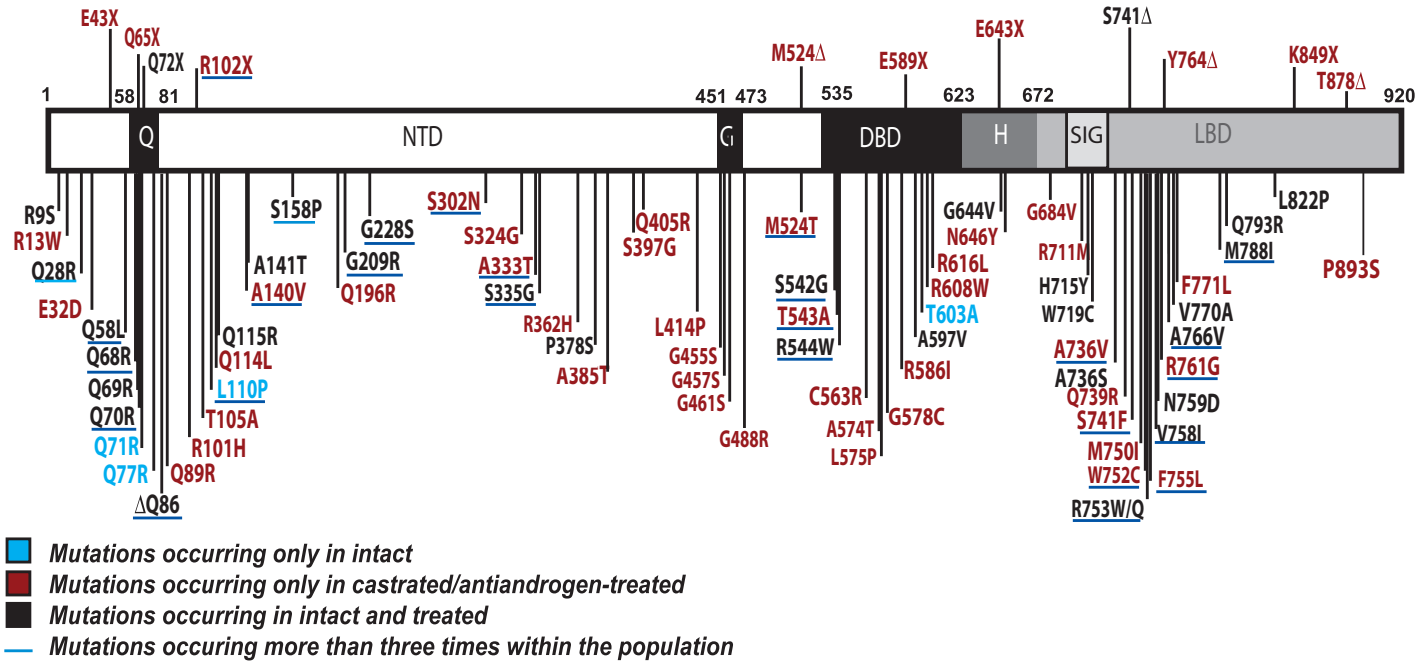


Fig. 4. A Treatment Schematic: Humanized AR mice expressing prostate-specific T-antigen (h/mAR X TRAMP) were randomly assigned to treatment groups (10 mice/treatment). Mice at 12 weeks of age, when PIN is just developing, were either castrated, treated with flutamide or biculatamide or left intact. Upon tumor detection (Status 1), intact mice either received no treatment or were treated with the antiandrogens bicalutamide (Bic) or flutamide (Flu). Endstage tumors were harvested and AR was amplified in 4 fragments from endstage tumors from cDNA (DBD and LBD) or DNA (N-terminal domain). Products were subcloned and 20 clones/ fragment sequenced (10 from 2 separate RT reactions for RNA).
B. Schematic illustrating where codons that were altered in a minimum of two tumors on sequence analysis of ARcoding sequence in all h/m ARXTRAMP mice. Codons highlighted in red indicate that the nucleotide change that occured at this site resulted in the exact same amino acid in all tumors.

APPENDIX



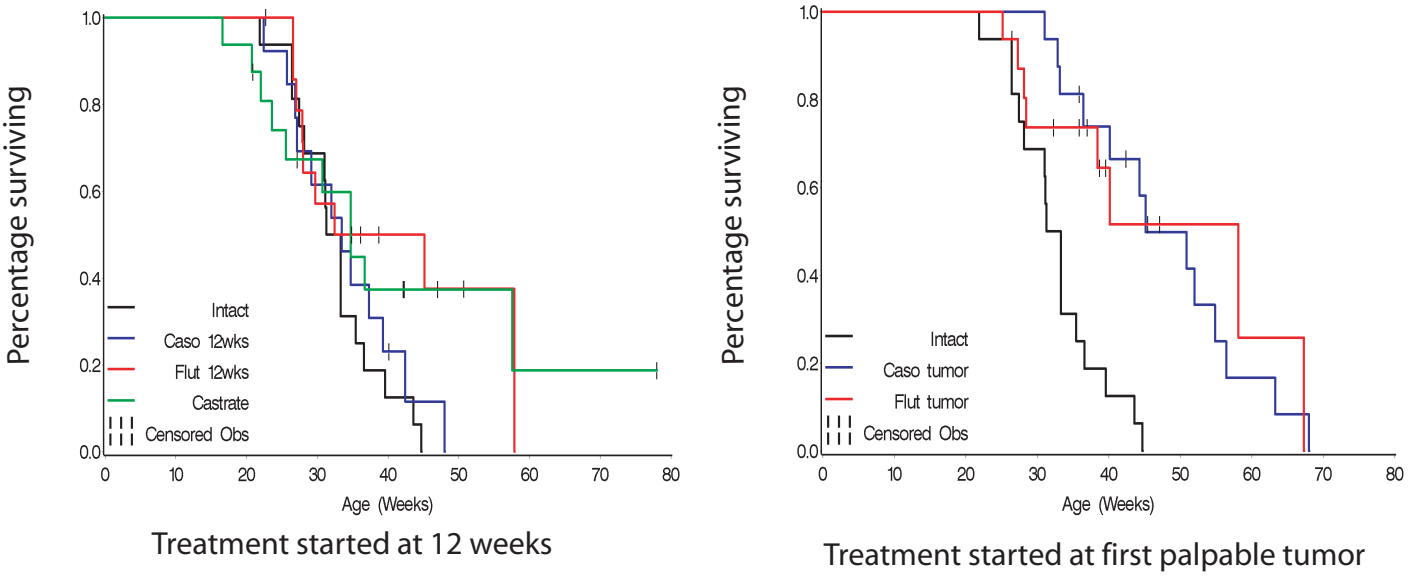
Mutations occurring only in intact
Mutations occurring only in castrated/antiandrogen-treated
Mutations occurring in intact and treated
Mutations occuring more than three times within the population

Figure 7 . A schematic of the AR protein and the amino acid substitutions identified in more than one h/mAR-TRAMP mouse tumor within the population. Underlined mutations occurred in three or more tumors. Δ: A base deletion occurred within the codon of the specified amino acid leading to a frameshift and subsequent premature stop codon.

APPENDIX

Survival Curves

A



Tumor Detection

B

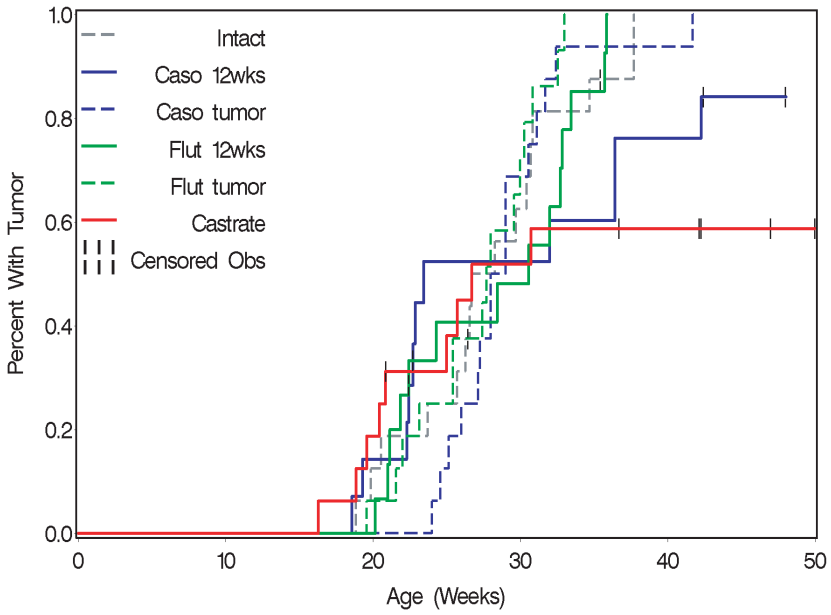


Fig 8 Kaplan -Meier methods were used to calculate and plot survival and tumor detection and cox models were used to assess differences between the groups. Survival time was defined as time from birth to death. Graph A1 shows survival curves of mice treated at 12 weeks with castration or antinadrogens. Graph A2 shows data for mice treated at the time of first detectable tumor.
Time to tumor detection is defined from birth to first detection of palpable tumor. Mice that did not develop a tumor were censored at the time of death. Graph B shows data for all non treated and treated groups at both time points.
Caso refers to bicalutamide treated mice while flu refers to flutamide treated mice.
Mice treated on tumor detection are descibed as either Caso tumor or flu tumor.

APPENDIX

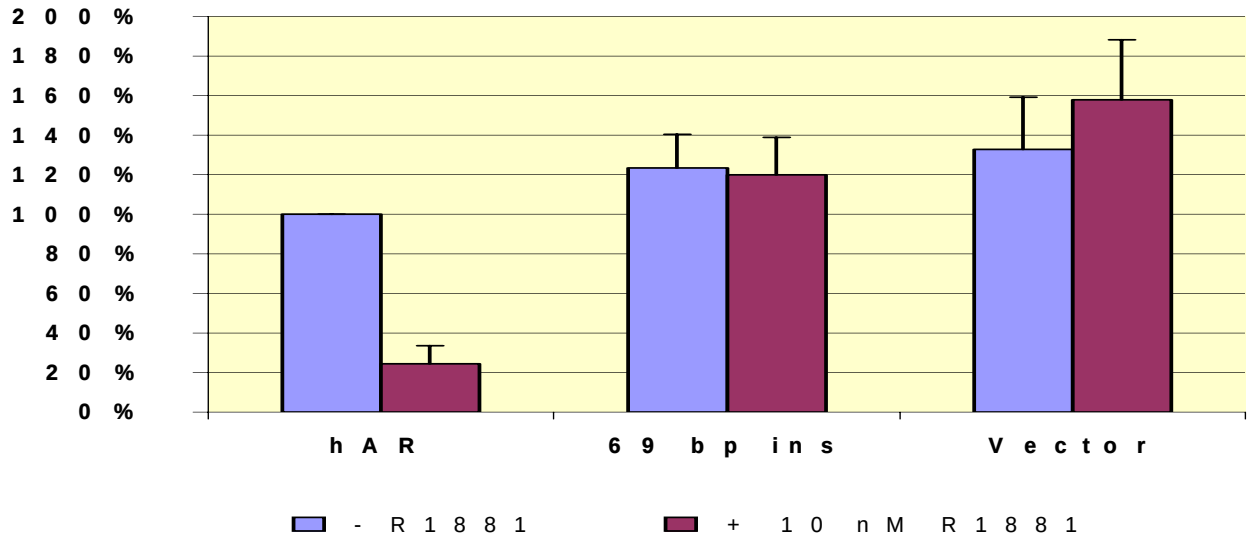


Figure 9-CV-1 cells were transfected with mutant or wild type AR and treated with 5 ng/ml TPA to induce NFkB expression and 10 nM R1881 to activate AR. The mutant 69bp insert was unable to repress NFkB transactivation. Data expressed as percent of wild-type activity.

APPENDIX

	Codon Change	Hormone Status
h/mAR-TRAMP	**Q58L**	Intact
	Q70R	Intact
	Q71R	Intact
	Q86 Deleted	Flutamide
	R102X	Flutamide
	G457G	Flutamide
	G467G	Flutamide
	G471G	Intact
	C564R	Flutamide
	A629A	Flutamide
	K849X	Flutamide
Xenograft	**Q58L**	Intact

Table 1: List of mutations (duplicated mutations) that were identified in two independent RT-PCR reactions.

Xenograft	aa	Codon	aa change	# of clones	Treatment status
	Y	11	C	2	Castrated
	P	12	P	2	Castrated
	P	12	S	2	Intact
	Q	58	L	2	Castrated
	Q	70	R	9	Castrated
	Q	86	deleted	2	Intact
	L	110	Q	2	Castrated
	L	145	P	2	Castrated
	P	152	C	2	Intact
	T	190	A	2	Intact
	Q	195	X	2	Castrated
	L	369	R	2	Intact
	G	453	D	2	Intact
	S	522	Del to X	2	Castrated
	L	595	del to X + 30 aa	2	Intact
	E	655	V	2	Castrated
	V	716	A	3	Intact
	V	747	del to X	2	Intact
	V	786	del to X	2	Castrated
	N	849	ins to X	2	Castrated
	V	867	M	4	Intact
	Q	86-91	INSERTION	2	Intact

Table 2: List of mutations identified in more than one plasmid per tumor on sequencing of the AR isolated from VCap xenogrfated tumors

h/m 21Qx TRAMP	aa	Codon	aa change	Sample identifier	#of clones	Treatment status
	R	13	Q	4948	2	Castrated
	R	13	W	4948	2	Castrated
	Q	58	L	4616	4	Intact
	Q	58	L	4243	2	Intact
	Q	58	L	4316	2	Castrated
	Q	58	L	5269	2	Casodex
	Q	70	R	4616	2	Intact
	Q	71	R	4616	2	Intact
	Q	86	6Q to 5Q	4530	3	Intact
	Q	86	6Q to 5Q	3829	2	Castrated
	R	102	X	5192	4	Flutamide
	T	105	A	4318	2	Castrated
	L	110	P	4243	2	Intact
	Q	114	L	4318	2	Castrated
	A	140	V	4724	2	Castrated
	G	209	R	4318	2	Castrated
	S	324	G	4316	4	Castrated
	A	385	T	4316	4	Castrated
	S	397	G	4318	2	Castrated
	M	524	T	5184	2	Flutamide
	M	524	T	5021	9	Flutamide
	T	543	A	4948	2	Castrated
	T	603	A	4616	2	Intact
	R	616	L	5021	2	Flutamide
	W	718	C	5021	2	Flutamide
	Q	739	R	5061	2	Flutamide
	W	752	C	4948	2	Castrated
	F	755	L	5021	2	Flutamide
	F	771	L	4948	2	Castrated
	Q	867	X	1698	2	Casodex
	P	14	silent	5059	2	Castrated
	I	34	silent	4318	2	Castrated
	G	51	silent	4318	2	Castrated
	Q	70	silent	1698	2	Casodex
	T	190	silent	4318	2	Castrated
	G	469	silent	4530	2	Intact
	G	578	silent	4021	3	Intact
	N	692	silent	5184	5	Flutamide
	N	692	silent	5200	5	Casodex
	G	709	silent	5184	5	Flutamide
	G	709	silent	5200	4	Casodex
	G		DELETION	5327	2	Flutamide
	Q	60	deleted	5184	3	Flutamide
	Q	63	deleted	5115	2	Flutamide
	Q	64	DELETED	5192	2	Flutamide
	Q	86	DELETED	5227	3	Casodex
	Q	86	DELETION	5247	2	Flutamide
	Q	86	DELETION	5247	2	Flutamide

	Q	91	DELETED	5227	5	Casodex
	Q	91	deleted	5115	3	Flutamid
	G	453	DELETED	5353	2	Casodex
	G	454	DELETED	5227	2	Casodex
	G	455	DELETED	5227	2	Casodex
	G	459	deleted?	1698	3	Casodex
	G	462	deleted	5115	2	Flutamide
	G	464	DELETED	5291	2	Casodex
	G	467	deleted	5246	4	Casodex
	G	472	DELETED	5208	2	Casodex
	G	473	DELETED	5208	2	Casodex

Table 3 List of mutations that were identified in multiple plasmids per tumor on sequencing the AR coding region isolated from h/m 21Q X TRAMP prostate tumors. Grey highlights silent mutations and deletions that were not included in Venn diagram analysis